2052-Pos Board B189
Real-Time Imaging Reveals that HIV-1 Vpr Dissociates from the Core and Accumulates in the Nucleus after Viral Fusion
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Viral protein R (Vpr) is an HIV-1 accessory protein that associates with capsids during viral assembly and is important for infections in non-dividing cells. Vpr functions in host cells include induction of G2 cell-cycle arrest, and regulation of cellular proliferation and apoptosis. Vpr has two nuclear localization sequences that direct its transport to the nucleus. Fluorescently labeled Vpr (YFP-Vpr) is widely used to visualize HIV-1 cores in the cytoplasm during entry. Here we report on the dissociation of YFP-Vpr from HIV-1 cores shortly after viral fusion and its subsequent accumulation in the nuclei. Real-time live cell imaging showed that, under conditions of productive entry and infection, Vpr dissociated from cores post-fusion and accumulated in nuclei over time-scales that correlated with the kinetics of viral fusion (t1/2~15 min). Nuclear accumulation of Vpr scaled with the number of cell-bound virions and could be blocked by lysosomotropic agents or a fusion-inhibitory peptide. These effects were observed in two cell lines and were independent of the fusion proteins incorporated into viral particles. Fluorescence recovery after photobleaching of YFP-Vpr within the nucleus revealed quick (t1/2~3 min) recovery, indicating that Vpr dissociation from capsids is a rate-limiting step in Vpr post-fusion transport. Fluorescence correlation spectroscopy measurements on post-fusion nuclear YFP-Vpr, yielded fast and slow diffusive components (D~10 μm2/s and 0.8 μm2/s, respectively) similar to those measured for YFP-Vpr over-expressed in cells. These diffusion coefficients reflect that nuclear Vpr exists in two forms - as a monomer, and in large complexes with host proteins or perhaps even chromatin structures. Current efforts are underway to explore the determinants of the stability of Vpr-capsid complexes.
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Delivery of Liposomal Contents to Outer Membrane Vesicles from Gram Negative Bacteria
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Gram negative bacteria produce small ~50-200 nm vesicles from their outer membranes. These outer membrane vesicles (OMV) have been implicated in activities such as transmission of virulence factors, horizontal gene transfer and development of biofilms. In this investigation, we continue our studies on the association and/or fusion of various liposomes with OMV. The delivery of large encapsulated molecules into OMV from *L. enzymogenes* C3 was investigated using liposomes with lipid compositions previously observed to be apparently fusogenic (Bartos et al., Biophys. J. 104(2) suppl1, 90a). Liposomes (100 nm) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) in a 1:3 ratio or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were used to encapsulate dextran conjugates of Texas red averaging 40 kDa. They were incubated with *Lysobacter* OMV (30°C, 1 hr.), then sedimented through 15% iodixanol, and the fluorescence monitored as indicatiive of transfer of liposomal contents to fused products. Both liposomal compositions showed significant evidence of dextran transfer. Because biofilms also contain OMV, the interaction of these liposomes with *E. coli* (DH10B) biofilms was also investigated via fluorescence microscopy. Significant penetration and binding within the biofilm mass was observed, as well as possible fusion with OMV, and rarely, evidence of transfer of dextran into whole bacterial cells. Fluorescence resonance energy transfer (FRET)-based assays also demonstrated that liposomes as small as 30 nm could rapidly fuse with *Lysobacter OMV*, suggesting possible delivery to OMV with smaller perturbation and better biofilm penetration.

2054-Pos Board B191
Fusion Fore Dilation by Snare Proteins
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Hormones and neurotransmitters are released through exocytotic fusion pores (FPs) that can flicker open and shut multiple times, fluctuate in conductance, and either dilate or reseal irreversibly. FP properties determine the size and the amount of cargo released, and the time course of release, which modulate downstream effects. Fusion is driven by formation of a four-helix complex between the neuronal/exocytotic vesicle-associated v-SNARE VAMP2/synaptobrevin-2 (VAMP2) and its cognate plasma-membrane t-SNARE partner composed of syntaxin-1 and SNAP25. Pore nucleation requires zippering between the v- and t-SNAREs, but what molecular factors govern the subsequent pore dilation is not understood. Here, using conductance measurements across voltage-clamped single FPs we show that multiple SNARE proteins cooperate to dilate metastable FPs. We isolated flickering fusion pores in a biochemically defined assay where v-SNARE-reconstituted bilayer nanodiscs (vNDs) fuse with cells ectopically expressing cognate, ''flipped'' t-SNAREs. Using newly developed, large NDs that are 21-27 nm in diameter, we varied v-SNARE copy numbers from zero to up to 11 per ND face. Pore nucleation required a minimum of 2, and reached a maximum above ~4 copies per face, but the probability of pore dilation was far from saturating at 11 copies, the maximum that the NDs could hold per face. Our results indicate that copy numbers of available SNAREs may be pivotal in determining whether neurotransmitters or hormones are released through a transient (kiss & run) or an irreversibly dilating pore (full fusion) and provide a rationale as to why synaptic vesicles carrying 70 copies of v-SNAREs dock onto sites where as many t-SNAREs are clustered while only a few SNARE complexes are apparently enough to achieve fusion.

2055-Pos Board B192
Control of Fusion Pore Nucleation and Dynamics by SNARE Protein Transmembrane Domains
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Membrane fusion is a fundamental biological process, whose initial stages have been observed in hormone-secreting cells and neurons using electrophysiological and electrochemical methods. The initial connection between the plasma membrane and a hormone- or neurotransmitter-filled vesicle -the fusion pore- can flicker open and closed repeatedly before dilating or resealing irreversibly. Pore dynamics determine events such as vesicle recycling and release kinetics, but pore properties are poorly known, because fusion pores are transient, and biochemically defined assays with single-pore sensitivity are lacking. We isolated single flickering pores connecting v-SNARE-reconstituted nanodiscs to cells ectopically expressing cognate, ''flipped'' t-SNAREs, voltage-clamped in the cell-attached configuration. Currents through such pores directly report sub-millisecond single-pore dynamics. We found that interactions between v- and t-SNARE transmembrane domains (TMDs) observed in a recent crystal structure promote, but are not essential for pore nucleation. Surprisingly, TMD interactions also affected pore lifetimes. Rod-shaped, post-fusion cis-SNARE complexes vacated the highly curved fusion site where they fit poorly, leaving pore properties to be determined largely by lipid bilayers. In contrast, Y-shaped mutants deficient in TMD-zippering lingered at the fusion site, preventing pore resealing for >60 s. Thus, post-fusion geometry of the proteins determines pore stability, analogous to the well-known effects of lipid geometry on highly curved fusion intermediates.

2056-Pos Board B193
Snare Mediated Fusion with Membrane Tension Control
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Fusion of membranes is ubiquitous in life. It is essential for neurotransmitter and hormone release, intracellular vesicular trafficking, fertilization, and viral infection. SNARE proteins constitute a highly conserved minimal fusion machinery mediating intracellular membrane merger from slow fusion of large yeast vacuoles (minutes) to extremely fast neurotransmitter release (<1 ms). While membrane tension was suggested to inhibit fusion by suppressing dimpling of membranes by viral fusion proteins (Markosyan et al., Biophys J, 1999), it was suggested to promote fusion pore opening and dilation in other studies (Shillcock and Lipowsky, Nat Mater, 2005; Nikolaus et al., Biophys J, 2010; Warner and O'shaughnessy, Biophys J, 2012). Thus, membrane tension may affect distinct stages of the fusion process differentially. To resolve how tension affects fusion, we established a fusion assay in which membrane tension is precisely controlled. Our approach is based on a previously established bulk assay in which v-SNARE reconstituted small liposomes (vSUVs) fuse to t-SNARE containing giant unilamellar vesicles (tGUVs, ~10-30 micrometers in diameter) (Malsam et al., EMBO J, 2012). Using a micropipette, a single GUV is picked up, whose membrane tension is controlled by the aspiration pressure. Another pipette is maneuvered nearby to puff a suspension of vSUVs. Fusion is monitored as an increase of the GUV tongue projection in the aspiration pipette whose position can be determined with sub-pixel resolution. Because the

micromanipulation setup is mounted on a spinning disc confocal microscope, simultaneous monitoring of fluorescence from labeled membranes can also be used to probe vesicle docking and fusion. Our preliminary results show increasing membrane tension increases the fusion rate.

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Collective Action of SNAREpins Exerts Forces between Membranes that Activate Fusion

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SNARE proteins mediate most intracellular membrane fusion processes such as exocytosis. It has been established that vesicle associated v-SNAREs and target-membrane associated t-SNAREs, assemble into a parallel four-helix bundle (SNAREpin) in a zipper like fashion, which brings the membranes close together. However, the detailed mechanisms whereby SNAREs drive fusion remains unclear. It is also unknown whether several SNAREpins cooperate to induce fusion, and widely varying SNARE requirements for fusion are reported, 2-15 in vivo (Montecucco et al., Trends Biochem Sci, 2005) and 1-11 in vitro (van den Bogaart et al., NSMB, 2010; Karatekin et al., PNAS, 2010). Here, we developed a mathematical model of SNAREpins connecting a vesicle to a planar membrane, quantifying inter-membrane, inter-SNAREpin and membrane-SNAREpin interactions, and taking account of the zippering/unzipping of the SNAREpins. Monte Carlo simulations showed that SNAREpins assemble through the SNARE motifs and self-organize into a ring. The ring tends to expand, driven by inter-SNAREpin and SNAREpin-membrane interactions, reducing membrane separation by geometrical coupling. Assuming an energy criterion for fusion, we determined the waiting times for fusion from the distributions of inter-membrane energies. Our data show that although one SNAREpin can induce membrane fusion, fusion waiting times decrease rapidly with the number of SNAREpins. Applying the model to single-vesicle fusion assays, we predict that the dependency of docking-to-fusion delay times on the number of v-SNAREs reaches a plateau, in agreement with experiments (Karatekin et al., PNAS, 2010). We also find that waiting times increase with vesicle size and with insertion of flexible segments into the SNARE linker domains, in qualitative agreement with experiments (McNew et al., Mol Cell, 1999). Our results suggest that fully zippered SNAREs work in concert to trigger fusion, and explain the wide range of reported SNARE requirements for fusion.

2058-Pos Board B195

Cholesterol Modulates SNARE Mediated Hemi- and Full-Fusion

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Cholesterol is essential for exocytosis in secretory cells, but distinguishing contributions from lateral organization and dynamics of membrane proteins and stabilization of fusion pores by intrinsic curvature and other mechanical effects of cholesterol have been elusive. The direct effect of cholesterol on fusion pore formation was examined between synaptobrevin 2 (VAMP 2) containing proteoliposomes and an acceptor SNARE complex containing planar supported bilayer using both membrane and content fluorescent markers. This revealed that increasing cholesterol in either the planar supported bilayer or in the synaptobrevin proteoliposome decreases the amount of hemi-fusion and increases the amount of full-fusion with minimal effects on the fusion kinetics.

2059-Pos Board B196

Chasing the Functional Asymmetry between C2A and C2B in Full-Length Synaptotagmin 1 during Ca^{2+}-Dependent Membrane Binding

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Synaptotagmin 1 (Syt1) acts as the major calcium sensor in neuronal exocytosis. Syt1 is a synaptic vesicle-anchored membrane protein that contains two tandem Ca^{2+} binding C2 domains, named C2A and C2B. There is evidence that Syt1 interacts with membrane lipids and SNARE proteins simultaneously to facilitate two key steps during Ca^{2+}-triggered membrane fusion: vesicle binding and content release. Although the overall function of Syt1 has been extensively investigated, the detailed molecular behavior of the single C2A and C2B domains in the neural regulatory process remains unclear. In particular, the differential function of the two C2 domains and how they cooperate in membrane fusion has not been determined.

Here we employed EPR spectroscopy, fluorescence interference contrast (FLIC) microscopy and total internal reflection fluorescence (TIRF) microscopy to dissect the state of C2A and C2B domains in full-length Syt1 during Ca^{2+}-dependent membrane binding. CW-EPR lineshapes and power saturation of spin-labeled positions in calcium binding loops of C2A in full-length Syt1 (1-421) and truncated Syt1 (1-266) without C2B domain suggest that C2A domain alone in Syt1 has a stronger membrane binding ability. A TIRF liposome capture assay further reveals that truncated Syt1 has a significant higher initial rate and extent in trans-binding with liposomes than full-length Syt1. Our data indicate that the C2B domain might be involved in some cis-binding thereby reducing total liposome binding. FLIC microscopy validates the strong involvement of C2A during Ca^{2+}-dependent liposome binding.
Our detailed structural and functional information thus provides a clue to differential regulatory mechanisms employed by C2A and C2B domains in full-length Syt1 interacting with Ca^{2+} and key membrane lipids, such as PS and PIP2.

2060-Pos Board B197

Mechanical Model for Self-Assembly of Synaptotagmin on a Lipid Membrane

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Synaptotagmin (Syt) is a calcium-sensor that is responsible for the action-potential-controlled fusion of synaptic vesicles to pre-synaptic membranes. Recent biochemical and structural studies show that Syt can form ring-like oligomers, which occasionally convert into tubular structures on monolayer or bilayer membrane with buckled membrane inside. This suggests certain mechanical interactions between the Syt and the lipid bilayer. To explore it in detail, we developed a coarse-grained mechanical model assuming that (i) Syt self-polymerizes into an elastic chain with a spontaneous curvature; (ii) Syt attracts the membrane through binding sites located at the inner side of the Syt molecule; and (iii) membrane is a uniform sheet with constant bending rigidity and tension. Using computer simulations, we have been able to estimate the spontaneous curvature and bending stiffness of the Syt chain. The model also allowed us to understand how the Syt oligomerization depends on the strength of Syt-membrane adhesion, the bending rigidity of the membrane, and the pressure on the membrane. These experimentally testable predictions from this modelling study will provide insight into the molecular mechanism of calcium-triggered membrane fusion at the synapse.

2061-Pos Board B198

Single Vesicle Assay to Study Membrane Tethering and Docking Factors

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In vitro vesicle-vesicle fusion assays based on fluorescence resonance energy transfer (FRET) of lipid or content mixing are widely used to investigate the molecular mechanism of membrane fusion process. However, without FRET signals from lipid or content mixing, these ensemble assays are insensitive to early stages of fusion such as tethering and docking. We have developed a single vesicle assay to study protein factors involved in tethering and docking [1-3]. Through our assay, we have studied proteins inducing membrane aggregation [4] and enhancing SNARE-mediated vesicle docking [5-6].
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2062-Pos Board B199

Deficiency of HID-1 Leads to Impaired Proinsulin Processing

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Diabetes mellitus has been a major social problem due to its prevalence, which results from a combination of peripheral insulin resistance and insufficient insulin secretion.The whole process of insulin biosynthesis, transportation, maturation and secretion is largely understood, however, detailed mechanism remains to be uncovered. We reported here a highly conserved protein, HID-1, functions during the correct processing of insulin. We generated a beta cell-specific conditional knock out mouse model of hid-1 gene. The knock out mice showed significant glucose intolerance while normal response to insulin with no significant defect in islets' morphology. Further study revealed remarkable increase in proinsulin to insulin ratioand abnormal proinsulin accumulation, suggesting that HID-1 may function in the conversion process from proinsulin to insulin.